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The University of Texas Medical Branch at Galveston

School of Medicine
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DEPARTMENT OF MICROBIOLOGY

September 8, 1989

Commander
USAMRDC
Attn: SGRD-RMI-S
Building 504
Fort Detrick
Frederick, MD 21701-5012

Dear Sir:

Enclosed is a revised copy of our annual report on the contract "Combination Chemotherapy Using Immune Modulators and Antiviral Drugs against Togaviruses and Bunyaviruses," Contract No. DAMD 17-86-C-6119. The report covers the period 3/1/88 - 2/28/89. The final version responds to corrections and requests of the technical and scientific reviews.

Sincerely,

Samuel Baron, M.D.
Chairman and Professor

SB/bs

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We have studied model arboviruses and arenaviruses to develop effective combination therapies using antiviral drugs and immunomodulators. The compounds were examined using Pichinde' (PIC) an arenavirus; Banzi (BZ) and West Nile (WN), two flaviviruses; Semliki Forest (SF), an alphavirus; and Bunyamwera (BW) and LaCrosse (LAC), two Bunyaviruses. Ribavirin was very effective against PIC, allowing for the development of lasting immunity to reinfection in surviving animals. Interferon (IFN) and IFN inducers had a consistent prophylactic antiviral action against all arboviruses tested, while not affecting PIC infection. The combination of ribavirin and IFN-α/β provided a synergistic antiviral effect against BZ, SF, and LAC; tumor necrosis factor (TNF) and IFN-α/β provided greater than additive antiviral effect against BZ; while the combination of interleukin-2 with IFN-α/β gave erratic results against the same viruses. Finally, successful combined therapy of SF or BZ infected mice was achieved with poly I:CLC and antibodies, when the intervention was started at the time of virus spread to the target organs. This treatment provided a strong, long lasting immunity in treated animals.

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Combination Chemotherapy Using Immune Modulators and Antiviral Drug Against Togaviruses and Bunyaviruses

ANNUAL REPORT

D.H. Coppenhaver, M. Sarzotti, I.P. Singh, H. Lucia and S. Baron

April 15, 1989

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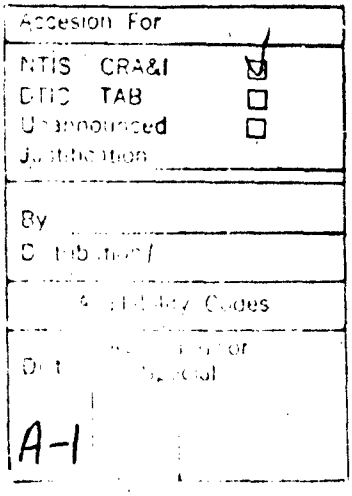

Summary

RNA viruses in the families Alphaviridae, Flaviviridae, Arenaviridae, and Bunyaviridae include many important human pathogens. In order to enhance our ability to prevent and treat viral diseases caused by these common pathogens, we have undertaken preclinical testing of candidate immunomodulators and antiviral drugs. The most promising of these antiviral agents were evaluated in bimodal combination treatments in vivo against Banzi virus, a flavivirus, Semliki Forest virus, an alphavirus, and, in some cases, La Crosse virus, a bunyavirus. Promising in vivo combinations include ribavirin with interferon or interferon inducers, interferon with tumor necrosis factor, and antibody with poly I:CLC. The latter combination also proved to be effective therapeutically against Banzi and Semliki Forest virus infections, even in a model which introduces virus intracerebrally before distal therapeutic treatment is begun.

Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or service of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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Introduction and Rationale

The research being pursued under this contract has the ultimate goal of designing and identifying effective therapeutic and prophylactic regimes for the treatment of medically and militarily important viral infections. Specifically, we are concerned with alphaviruses, flaviviruses, bunyaviruses, and arenaviruses. It was the primary goal of the initial contract year to establish test systems *in vitro* and *in vivo* in order to investigate antiviral agents against representatives of each of these virus groups. In the second and third contract year we concentrated our efforts on exploring possible combination antiviral therapies *in vivo*, using immunomodulators and antiviral compounds which we identified as the most promising candidate antivirals, based on single drug protection experiments. We have focused *in vivo* work in the second and third contract years on three virus systems: Pichinde' (PIC), an arenavirus; Banzi (BZ), a flavivirus; and Semliki Forest (SF), an alphavirus. In some cases, the results from these virus systems have been extended and confirmed with tests using West Nile (WN), a flavivirus; La Crosse (LAC), a bunyavirus; or Bunyamwera (BW), a second bunyavirus.

Primary evaluation of combination antiviral therapy was in the short-term prophylactic mode, with treatment beginning immediately prior (1 hr - 2 days) to viral challenge. Further evaluation of promising antiviral combinations was also undertaken therapeutically. In these studies we attempt to achieve late therapy by starting intervention at the time of viremia, when the virus is beginning to spread to target organs. The state of viremia was selected for the onset on therapy because it causes the first identifiable symptoms of disease that in humans would bring the patient to a physician for treatment. In these experiments, using murine models, the state of viremia is identified by detection of both virus and accompanying clinical correlates of infection, such as fever and weight loss.

Experimental Methods

Viruses. Seed alphaviruses [Semliki Forest (SF), Sindbis (SB)], flaviviruses [Banzi (BZ), West Nile (WN)] and bunyaviruses [Bunyamwera (BW), LaCrosse (LAC)] were received from Dr. Robert Shope of the Yale Arbovirus Unit. *In vivo* experiments were performed using SF (strain original, passage 15), BZ (strain SA H336, passage 11), West Nile (strain Egypt 101, passage 18), Bunyamwera (strain original, passage 53) and La Crosse (strain original, passage 5). Pichinde' virus was received from Dr. David Gangemi, University of South Carolina. The virus (0.1 ml) was inoculated i.p. into 450-500 g Strain 13 guinea pigs. The animals were sacrificed on day 6 post infection, and 10% spleen suspensions prepared. This virus suspension was repassaged in Strain 13 guinea pigs to prepare the final stock virus (passage 14), which was aliquoted and frozen (-70°C).

We noted that after 6-9 months of storage at -70°C, virus stocks begin to lose pathogenicity *in vivo*. Because of this BZ and SF viruses are routinely repassaged in newborn mice. Briefly, viruses were inoculated intracranially into suckling mice 1-2 days postpartum. At the first sign of illness, generally 2-4 days after virus challenge, the mice were sacrificed and a 10% brain suspension in Eagle's minimum essential media was prepared. The 10% brain suspensions were aliquoted and frozen (-70°C) for future use.

Interferon and Interferon Assay. Natural murine interferon (IFN) α/β was produced in our laboratories by a modification of the method of Dussaix et al. (J. Gen. Virol. 64:285, 1983). Briefly, L-929 cells are grown to confluency in roller bottles at which time they are incubated (37°C, 18 hr.) with 50 ml Eagle's minimum essential media (EMEM) containing 0.22% bicarbonate, 0.1% penicillin-streptomycin, 2 mM arginine butryate, 1 mM theophylline, and 5% fetal bovine serum. After 18 hr. the cells are decanted and inocuated with Newcastle Disease virus in EMEM and incubated with rolling for 3 hr. At the end of this incubation, the cells are decanted and washed with Hank's balanced salt solution before the addition of 50 ml EMEM containing 0.22% bicarbonate, 0.1% penicillin-streptomycin, 292 µg/ml L-glutamine, and 1 mM theophylline. After 24 hr. the IFN containing supernatant is harvested. The supernatants are stored at pH 2 (4°, 96 hr.) to inactivate any IFN-µ before neutralizing, aliquoting, and storing at -70°C until use.

All interferon (IFN) preparations were titrated in our laboratory by a plaque-reduction assay in microtiter plates with L-cell and vesicular stomatitis virus as described in our previous reports. Our laboratory unit/ml of IFN was defined as the concentration of IFN which reduced the number of virus plaques to 50% of the control level. In our system, one laboratory unit corresponds to one international reference unit (IU) (NIH standard G002-904-511). Activity of IFN preparations in guinea pigs was evaluated using an analogous system, except that the assays were performed using guinea pig embryo cells.

Production of rabbit anti-virus antibodies. Antibodies to Banzi and Semliki Forest viruses for use in the late therapy model were prepared in rabbits, using a method to minimize the presence of non-virus directed antibodies. Virus was grown in 75 cm^2 T-flasks on RK-13 rabbit cells, in EMEM containing 2% rabbit serum and no fetal calf serum (RK-13 cells must first be adapted for growth in rabbit serum). After 24 hr virus yield is maximal. Cells are frozen (-70°C) before use. Frozen virus preparations are thawed and centrifuged to remove all debris. The virus-containing supernate is inactivated with β-propriolactone (0.2%, 37°, 2 hr), and mixed with Freund's complete adjuvant (1:1). Inactivated antigen (1-2 ml/rabbit) is injected in New Zealand white rabbits subcutaneously. Rabbits are boosted weekly with one ml of live virus preparation (i.v.) for three weeks, after which antibody containing sera are harvested at weekly intervals. Antigen boosters are given at monthly intervals thereafter.

Animal Models. Protection against arbovirus infections is evaluated in vivo using weanling mouse models. Female outbred mice, strain ICR, at 18-22 g (4-5 weeks) are inoculated with the drug to be evaluated. Route and schedule of drug delivery are depending on the specific compound being evaluated. Virus challenge (1-3 LD_{75}) is administered intraperitoneally (i.p.). Primary evaluation is by presence of an amelioration of lethal encephalitis. Mice are generally maintained for 14-20 days after virus challenge before an experiment is terminated, but all survivors are held for at least 3 days after the last recorded death or illness. Virus control animals die between day 7-9 (BZ), 5-7 (SF) 7-10 (WN), 8-11 (LAC), or 8-10 (BW) after virus challenge. Mortality in the LAC and BW system was erratic, varying from 20-100% of challenged mice in repeat experiments with a given virus inoculum. Pichinde' virus is administered i.p. to 450-500 g strain 13 guinea pigs. Primary evaluation is by amelioration of lethal hemorrhagic fever. Guinea pigs are maintained for 28-75 days after virus challenge. Virus control animals die between 13-19 days of virus

challenge. Statistical evaluation of individual experiments is by student's t, chi-square, or Fisher's exact test. Significant results are noted in the tables.

Results

A. Pichinde' virus infection in strain 13 guinea pigs.

Treatment of Pichinde' Virus Infection with Interferon and Interferon Inducers. Recombinant leukocyte alpha (A) interferon (Hoffmann-LaRoche) is known to be active in guinea pigs, and was used in these experiments. The activity of the rIFN-αA was calculated and compared to the activity in the human system, as reported by the manufacturer. One unit of activity in the guinea pig cell system was equal to 18 U of human activity (mean of 4 experiments).

Previous experiments with 30,000 U and 10,000 U (human units) of rIFN-αA showed no protective effect against Pichinde' virus. With the knowledge that guinea pig efficacy was only 1/18th of the human, we repeated these experiment using 3×10^6, and 3×10^5 human units. These are equivalent to 166,667 U, and 16,667 U, respectively, in guinea pig activity.

Twelve guinea pigs, weighing 450 gms. were divided into three groups, a high dose group, a low dose group, and the virus only group, which served as the control. The interferon was administered i.p. one hour before 0.1 ml stock virus was injected. No beneficial effect on weight loss or reduction in mortality was observed. A trend toward earlier death in the group receiving interferon was present, but not significant. Thus, at levels ranging from 10^4 - 3×10^6 I.U. (5.6×10^2 - 1.7×10^4 guinea pig units) no significant effect of IFN-α on PIC infections were observed (Table 1).

We have also evaluated the IFN inducing agent CL246,738 in the PIC system. We previously reported the results of a series of experiments, which can be summarized as follows: drug treated animals received either low dose (5 mg/kg) or high dose (15 mg/kg) of CL246,738 injected i.p. 24 hours before virus inoculation. Control groups received virus only, or drug only. All infected animals treated with CL246,738 died between days 14 and 16 after infection. Mean day of death for animals receiving 15 mg/kg was 14.4, for those receiving 5 mg/kg 14.6, and for untreated infected animals 15.7. Uninfected animals receiving drug appeared healthy, and gained 1% over initial body weight by day 14 (Table 2).

We next evaluated whether these results were due to a possible lack of activity of the CL246,738 in the guinea pig system. Strain 13 guinea pigs were injected with 2.5 and 7.5 mg/kg of CL246,738. Serum obtained at 6 hrs., 12 hrs., and 24 hrs. was tested for interferon activity in guinea pig cells infected with VSV. No IFN activity could be demonstrated. Spleen cells and peritoneal macrophages obtained at 6 and 24 hrs after inoculation with 7.5 mg/kg were cultivated in vitro, using standard techniques. No IFN activity could be demonstrated from the supernate of spleen cells. However, IFN was detected in the supernates of peritoneal macrophages obtained both 6 and 24 hours after administration of CL246,738. These results are similar to our results in the murine system, and demonstrate that the failure of CL246,738 to protect against PIC is not because of inactivity of the drug.

Rimantidine Treatment of Pichinde' virus Infection. Strain 13 guinea pigs infected with Pichinde' virus were treated with rimantidine, 3.0 mg/kg., on days 0, 1, 2, 3, 4, 7, 9, and 11 after infection with the standard stock Pichinde' virus inoculum containing 10^4 PFU. All infected animals died. However, the mean day of death for the control animals was 12.5, and for the drug treated animals 15.2, for a USAMRIID virus rating of 1.22. (Fig. 1). This indicates that rimantidine probably has some antiviral activity in this system, and may well be useful either in increased doses, with continuous therapy late in the infection, or in combination with other antiviral agents. In contrast, no effect of rimantidine was seen in bunya- or togavirus system (data not shown).

AM-3 Treatment of Pichinde' virus Infection. The immunomodulator AM-3 had been determined by us to be ineffective in preventing encephalitis induced in mice by alphaviruses and flaviviruses (see below). Reports that this agent was extremely effective against the hemorrhagic strain of Punta Toro bunyavirus led us to investigate its effect on a hemorrhagic arenavirus infection. AM-3 [25 mg/kg/day] was administered i.p. to strain-13 guinea pigs daily for five days, starting 24 hr before virus challenge. No protection from Pichinde'-induced mortality was seen in these experiments, and the weight loss in drug treated animals was parallel to, and slightly more severe than, that recorded in the virus controls. Mean day of death did show a nonsignificant increase in the treated animals, however [MDD AM-3 group = 14.2; Virus Control group = 13.7]. The USAMRIID virus rating of AM-3 in this system is 1.03.

Extended Ribavirin treatment of Pichinde' infection and resistance to reinfection. We have previously reported that extended ribavirin treatment was significantly effective in protecting PIC infected guinea pigs. Because of the relatively short half life of ribavirin, we investigated a twice daily treatment regimen. Animals received ribavirin i.p. 50 mg/kg/dose either once or twice a day, for thirty days after challenge with 10^4 PFU PIC. Virus control animals showed the expected 100% mortality, with an MDD of 13.0. The once daily treatment group (50 mg/kg) also showed 100% mortality (which was significantly higher than in previous experiments), but with MDD extended to 18.7. The twice daily treatment group (50 mg/kg dose; 100 mg/kg/day) had reduced mortality (66.7%) and extended MDD of 21.7. A summary of the effect of extended ribavirin treatment on Pichinde' infections of guinea pigs is given in Table 3.

We also investigated the development of natural immunity in animals surviving PIC infection as the result of ribavirin therapy. Three animals, which were infected with PIC and survived after a 28 day treatment with 50 mg/kg/day ribavirin, were rechallenged 3 months later. Control naive animals received the same virus inoculum. Control animals had 100% mortality by day 13 p.i. All rechallenged animals survived the infection, showing only a slight weight loss on day 13 p.i. Blood from these animals was drawn, and will be used to determine antibody and virus levels.

A second series of rechallenge experiments was conducted on two guinea pigs which were originally infected on with PIC, and survived after a 28 day treatment with 50 mg/kg/day ribavirin. These animals were rechallenged 3, 6, and 11 months later. No loss of weight or mortality was recorded in any of these rechallenge experiments. These two animals were sacrificed 1 year post-primary infection, after developing pneumonia. Autopsy showed flavibacterial bronchopneumonia, unrelated to PIC infection. Based on these

experiments, it appears clear that animals surviving PIC infection develop a lasting immunity to reinfection.

B. Immunomodulator and Antiviral Drug Titrations Against Alpha-, Flavi-, and Bunyavirus Infections of Mice.

Single drug dose response experiments using the murine model systems were run to confirm previous results and to obtain complete dose - response information in our model systems for agents to be used in combination experiments. It is particularly important to obtain information on the end points of single agents which might be used in combination trials so that levels of the antivirals which maximize the likelihood of detecting combinatorial effects are chosen. Throughout these experiments, the mortality induced by LAC, BW, and WN pathogenesis was somewhat erratic and less than ideal, particularly in the bunyavirus systems. For example, control LAC mortality with the same virus innoculum ranged from 20-100% in different experiments (Tables 4,6,7,9,11). We report protection data obtained in all cases relative to virus controls. We recognize that simple percentage reduction of mortality could be somewhat misleading when greater than 50% of untreated mice fully recover from the infection, however. Hence, we include the raw mortality data in the tables. In all cases when untreated control mortalities are low in a single experiment, protection recorded in the treatment groups can only be regarded as suggestive.

Interferon. We previously reported that natural murine IFN-α/β was a potent antiviral in the SF and BZ systems. We extended these studies to the WN, LAC, and BW system to test the generality of our previous findings. Results for natural murine IFN-α/β are given in Table 4. In the WN system, moIFN-α/β gave significant but erratic protection. Titrations of natural murine IFN-α/β in the two bunyavirus systems were much more regular than the results obtained in the WN experiments, with complete protection from lethal infection recorded with a single 100,000 to 300,000 unit dose in three independent experiments. These viruses appear to be closer to the IFN-sensitivity of BZ virus than they are to the relatively more sensitive SF system. Parallel titration of rHuIFN-α A/D in the WN system failed to protect mice at any level used (300 - 10,000 U/mo; i.p., 2 hr before virus challenge. Similar titrations of rHuIFN-α A/D in the SF model were carried out. Doses of the rHuIFN-α A/D needed to achieve levels of protection are comparable to those found earlier with natural murine IFN-α/β (Table 5). In a concurrent experiment, there was significantly less protection seen when four injections of rHuIFN-α A/D were given on days 0, 1, 2, and 3 than when only a single injection was given on day 0 (Table 5).

We have reported that the in vivo antiviral effect produced by IFN-γ is much less powerful than that induced by IFN-α/β. In most of these experiments, the IFN preparations are evaluated in the same treatment schedule. Since IFN-γ is known to produce an antiviral effect in vitro through a two step process, which is slower than the single round of protein production seen for IFN-α/β, we evaluated the effect of varying the time of administration of IFN-γ in the SF system. Both natural and recombinant murine IFN-γ were tested in this manner. No effect of the natural IFN-γ preparation was seen in these experiments (data not shown). Only a slight, nonsignificant antiviral effect was seen for rmo IFN-γ at the earliest times [-6,-18 hr.] and highest dose [3000 U/mo] used. Thus, while IFN-γ may indeed by slightly more effective when administered significantly prior to virus challenge, it is a much less effective antiviral than is IFN-α/β in these systems.

Interferon Inducers. We also evaluated the IFN-inducers poly I:CLC and CL246,738 in the WN, LAC, and BW systems. Significant protection in all three systems was recorded for poly I:CLC, although smooth dose response effects were not uniformly seen (Table 6). CL246,738 also appeared to be highly effective in the bunyavirus systems (Table 7).

For both poly I:CLC and CL246,738, the lowest levels used were doses at which only partial protection was seen in the togavirus systems. These results indicate that these IFN inducers are even more effective in treating infections caused by members of the Bunyavirus group than is the case for alphavirus and flavivirus mediated diseases. The universality of this observation is unknown at present and is in contrast to the results of IFN titrations, summarized above.

Ge-132 (carboxyethylgermanium sesquioxide) has been reported to be an orally active inducer of IFNγ in mice. We have run a series of experiments in both inbred (NSF, BALB/c, AKR) and outbred (ICR) mice to determine the level of IFN induced by the compound. Ge-132, supplied in the dry form by the ASAI Germanium Research Institute, was dissolved in phosphate buffer or Hank's balanced salt solution and given to weanling mice orally, i.p., or i.v. Results of a typical experiment are shown in Table 8. Ge-132 induced a high level of circulating IFN 24-48 hr after treatment in about half of the mice tested when administered at a level of 100-300 mg/kg. Lower levels of the compound were ineffective. There were no significant differences in the response using different routes of administration. Ge-132 also gave extremely erratic results in protecting mice from virus infection. Experiments with the liquid form of the drug supplied by USAMRIID showed no difference in percent mortality, MDD, or VR when 0.1 ml (5 mg/mo) was administered, either per os or ip, daily for 6 days, starting 2 days before SF virus challenge (data not shown). Similar experiments with the related inducer Ge-089 also failed to show significant protection. Neither low (0.1 mg/mo per os) nor high (10 mg/mo per os) doses of Ge-089 produced significant protection against SF. Alternate routes of administration were evaluated in the BZV system. A slight, nonsignificant effect was recorded only when the drug was given per os. This protection was not significantly greater than that recorded in the carboxymethyl cellulose control, however. Ampligen, a known inducer of IFN-α/β, which we have shown to be an effective antiviral in the BZV system, was included as a positive control in the later experiment, eliminating the possibility that the negative results were caused by unresponsive mice. Based on these results, we can see no consistent significant effect of this family of compounds on protection from togavirus infections or on the production of interferon in the mouse model.

Immumodulators. We have also been interested in evaluating immunomodulators which have important modes of action outside the IFN loop, in order to identify appropriate candidates for combination therapies with IFNs and IFN inducers. To this end non-encapsulated muramyl dipeptide (MDP) and muramyl tripeptide (MTP-PE) were evaluated in the Semliki Forest (SF) and Banzi (BZ) systems. Neither of these compounds showed any protective effect against lethal encephalitis in these systems. Since MTP-PE is known to be highly labile in the body, we reevaluated this comound using a liposome encapsulated formulation. Our first experiment, using SF, showed partial protection at the highest dose when two injections of the compound were given [30 µg/mo; -48, 0 hr]. A confirmatory series of experiments in SF and BZV gave much less positive results, however. No protection was recorded at the 10 and 30 µg doses, while

only 50% protection from lethal encephalitis was recorded even at a 100 µg/mo dose, and only in the BZV system. More problematic, however, was the partial protection given by the liposome control in these experiments. No drug treatment groups were significantly different from the liposome controls. In light of these results, it is doubtful whether MTP-PE can be considered an effective candidate antiviral for alpha- and flaviviruses, although it is possible that changes in the treatment schedule could produce more favorable results.

The immunomodulators AM-3 (AVS-1767) and mannozyme (AVS-1779) were also evaluated in the SF and BZ systems. No consistent protection was seen with AM-3 when a single i.p. dose (0.1 - 10.0 mg/mo) was administered 4 hr before virus challenge in either system. Unexpected difficulty in dissolving this agent in HBSS led us to repeat the experiment with the drug suspended in 0.4% carboxymethylcellulose (CMC). This procedure also gave negative results. Similarly, mannozyme (0.01 - 1 mg/mo) failed to protect in either system when given 4 hr before virus challenge. These results may be consistent with the reported difference in effectiveness of these compounds between encephalitic and hemorrhagic strains of Punta Toro virus (Sidwell, personal communication).

Antibodies and Antiviral Drugs. Two experiments with a milk-derived tissue fluid CVI preparation (Enf CJD 38) gave differing results in the LAC and BW systems. While no protection was seen against BW, significant protection was seen with a single dose of the milk-derived CVI in the LAC system (Table 9). This is in good agreement with the results of tissue culture experiments, which showed LAC to be more sensitive to the action of this inhibitor than is BW.

Since antibody is known to be protective against alpha- and flaviviruses, we devised an experiment to test whether polyclonal activation of B cells *in vivo* would protect against virus challenge. As a polyclonal activator we selected goat anti-mouse µ chain [$F(ab')_2$ fragment]. We elected to use $F(ab')_2$ fragments in order to avoid the possibility of complement mediated lysis of antibody coated B cells. Two doses of antibody fragments [0.002 - 20.0 µg/mo/injection] were administered 48 and 24 hr. before virus challenge. Slight protection from BZV was seen at the highest doses used in these experiments. To determine whether the anti-µ Abs was indeed capable of activating B cells, we tested the $F(ab')_2$ Abs for their ability to induce proliferation of splenocytes. Splenocytes from weanling ICR mice were incubated at 10^5/well in a microtiter plate for 48 hrs in the presence of $F(ab')_2$ or lipopolysaccharide (LPS, a positive control). The wells were pulsed for 18 hrs with ^{3}H-thymidine, and counts determined. Goat anti-mouse µ chain $F(ab')_2$ Abs induced a 4-5 fold increase in the proliferation of the splenocytes, and LPS a 9-fold increase (data not shown).

Based on these results, anti-µ $F(ab')_2$ Abs were retested *in vivo* against Banzi virus. Using the protocol described above, we increased the maximum dose from 20µg/injection to 40 and 80 µg/dose in these experiments. Results are shown in Table 10. Significant protection was seen in the first of these series of experiments, only marginal protection in the second, while no detectable protection was recorded in the third. No evidence of elongation of MDD was recorded in any of these experiments. The erratic nature of these results is puzzling, and could be due to inefficient delivery of the Ab, or to the use of insufficient Ab.

C. Combination antiviral treatment with ribavirin and interferon or interferon-inducers.

In our previous annual report, we summarized a series of investigations on the use of ribavirin in combination with IFN or IFN inducers. In all experiments, ribavirin was given as five daily injections, i.p., starting 24 hours before virus challenge. During the course of the experiments we determined that 800 µg/day (40 mg/kg) is the optional ribavirin dose in our murine model system. In the BZ system, the combination of ribavirin with natural murine IFN-α/β consistently produced a greater than additive protection against lethal encephalitis at IFN doses ranging from 10,000 to 30,000 U/mouse (i.p., 2 hours before virus challenge). This trend was also seen in the SF system. Further experiments confirmed the positive interaction in the SF system, and extended the observation to the LAC system (Table 11). In all of these experiments, it appears that the level of IFN administered must be sufficient to give a measurable response before any combination effects can be detected.

In previous experiments, we did not see positive interaction of ribavirin and mo IFNγ (1,000 - 3,000 U/mo) in the BZ system. Similar results were seen against SF at the same levels of IFN. Positive interaction was recorded when moIFN-γ levels were raised to 4 injections of 10,000 U/mo, however (data not shown). It may be that higher, and more continuous levels of IFN-γ are needed for significant enhancement of the virostatic action of ribavirin.

We have commented on an unexpected negative interaction of poly I:CLC with ribavirin when tested against BZ. In repeat experiments, this negative interaction was not significant, although there was clearly no evidence of enhancement with these two agents (data not shown). Against SF, however, slightly enhanced protection was consistently achieved with combinations of ribavirin and poly I:CLC (Table 12).

As an independent evaluation of the effect of ribavirin in combination with IFN inducers, this drug was examined in combination with CL246,738 in the BZ system. Moderate, but nonsignificant, enhancement of protection with lethal encephalitis was noted in preliminary experiments. Repeat experiments with BZ confirmed this positive interaction, while the effect was not observed in the SF system (Table 13). Preliminary evaluation of this combination in a bunyavirus system was uninformative, due to the level of protection recorded in single drug controls (Table 13).

D. Combination Antiviral Treatment with Lymphokines and Cytokines.

We continued studies on the use of combinations of lymphokines and cytokines in antiviral therapies, concentrating on combining IFN with tumor necrosis factor (TNF) and interleukin-2 (IL-2). TNF was administered i.p., daily for five days, starting 24 h before virus challenge. We reported that the combination of the rHuTNF-α with IFN-α/β consistently increased the MDD, and, at higher doses of TNF (1-3 ug/dose) significantly enhanced protection from BZ lethal encephalitis over the expected additive interaction of single drug controls. We investigated the mechanism of this interaction, and found that the combinations of rHuTNF-α (3ug/mo/day) with Mo IFN-α/β (10,000 U/mo) significantly reduced BZ viremia compound to virus and single drug controls (Figure 2).

Hydrazine sulfate has been reported to block the cachectic action of tumor necrosis factor (TNF). In preliminary experiments, it has been shown that this compound markedly potentiates the antiviral action of TNF against vesicular stomatitis virus in vitro. We tested whether this enhancement of the antiviral action of TNF by hydrazine sulfate was demonstrable in vivo. Using doses of TNF that are well below the antiviral level which we have already shown, there was no positive effect of either drug or combination of drugs against BZ encephalitis (data not shown). A maximum tolerated dose of hydrazine sulfate was used in these experiments; it may however be possible to duplicate the in vitro effect by using higher doses of TNF. Our reported interaction of TNF with IFN-α/β would also not be visible at the TNF levels used in this experiment; it is possible that all antiviral effects of TNF require a large dose to be visible.

Experiments on the interaction of IFN-α/β and IL-2 were continued. IL-2 was given i.p., 24 hrs before virus challenges, while IFN was given i.p. 2 hrs before virus challenge. We previously noted a significant positive interaction of these lymphokines against BZ. A series of experiments against BZ and SF infections produced extremely erratic results. At present, we are unable to reliably reproduce a synergistic antiviral effect of these two compounds.

E. Post-infection therapy of BZ and SF infections

Using our model of late intervention, after the onset of clinically detectable correlates of viral infection, we continued studies on therapeutic antiviral treatment. We have concentrated our efforts with the late intervention model on therapy using antibodies and the interferon-inducing immunomodulator poly I:CLC. When 1-5 daily doses of poly I:CLC (80 μg/mo) are given to fevered mice starting on day 3 post-infection, no significant protection from BZ lethal encephalitis was recorded, although a single prophylactic dose was highly protective and a combination of prophylactic and therapeutic treatments were completely protective in the same series of experiments. Single day 2 interventions in the SF model were similarly unsuccessful. Consequently, for the first series of experiments, a single dose of rabbit antibody against challenge virus and a single dose of poly I:CLC (80 μg/mo) were administered at a clinically relevant time point after virus challenge.

We previously reported evidence, using murine anti-BZ antibodies, that the combination of antibody and poly I:CLC was effective at preventing lethal encephalitis when administered 72-96 hr after virus challenge. Repeat experiments with rabbit anti-BZ confirmed this finding (not shown). An analogous protocol was used in treating SF infections. As we have done in the BZV model, we chose the combination of 1) polyclonal rabbit antibodies directed against the challenge virus and 2) poly I:CLC. Intervention was attempted at 8 to 72 hrs after virus challenge. In the SF system, significant viremia is present 24 hr post-infection, and virus has reached the brain by 48 hr post-infection. In control experiments, a demonstrable anorexia (weight loss) was seen in about 60% of infected mice, while a smaller percentage also showed an elevated temperature. In the current experiments, however, these clinical correlates of infection were only evident in <2% of the mice. Because of this, mice were treated regardless of the presence of fever or anorexia. All combination treatment mice received a single 80ug dose of poly I:CLC at the indicated time. The most remarkable results were recorded at the lowest doses of anti-SF tested. Although neither poly I:CLC nor 500 U anti-SF alone gave any protection from

SF-induced lethal encephalitis, the combination of these agents produced a 70% survival rate. Enhanced protection was also recorded with 80ug poly I:CLC plus 1000 U anti-SF. Delay of intervention until 72 hr post-infection, by which time the virus has replicated to high titers in the brain, abrogated the protective effect of this combination treatment. In repeat experiments, moderate antibody levels (500-1000 U/mo) were totally protective at 8 hr p.i.; protection decreased thereafter. Enhanced protection by the combination treatment was particularly marked at 24 hr p.i., which represents the time of peak serum virus levels in the SF infection. Enhancement of Ab protection by poly I:CLC was less marked at 48 hr p.i., when virus is reaching the brain (Table 14).

Because of unavoidable uncertainty in determining the progression of the virus to the brain in individual animals, it was decided to investigate the efficacy of this combination treatment after SF virus is in the target organ by using intracranial (i.c.) virus challenge. Treatments are administered i.p., distally from the virus challenge site. Table 15 shows the effect of varying the time of treatment after i.c. virus challenge. Protection was recorded at all times tested, from -15 hr to +24 hr p.i. There was no clear effect of the time of treatment established. Because maximal protection seemed to occur with treatment at +6 hr, this time was chosen for the subsequent series of experiments. Infection of the animals by the i.c. route has the advantage of assuring that virus has reached the target organ in all of the experimental animals, thus avoiding the possibility that animals rescued by late therapy represent those animals in which virus had not yet reached the brain. Starting therapy at +6 hr relative to the infection allows time for the first viral replication cycle to occur, further assuring that the infection is well established before therapy at the peripheral site is started.

In two sets of experiments (Table 16), mice were treated with 3LD75 SF i.c. (virus inocula via the i.c. route were determined to be 30% of the corresponding i.p. dose needed to produce the equivalent pathogenic effect) and subsequently treated with 80ug poly I:CLC, 1000 or 2000 U anti-SF Ab, or both, at +6, or +6 and +96 hr by the i.p. route. In all cases, no significant protection was recorded by single or double individual drug therapy. All cases of combined therapy gave significant, synergistic protection against the lethal SF encephalitis, however. The double dose (+6, +96 hr) treatments tended to be slightly more effective than the +6 hr combinations (Figure 3). When the same experiment was run in the BZV system, slightly less impressive combination protection was found. No additional protection was seen by the addition of one or two doses of poly I:CLC to the +6, +96 hr Ab treatment (Figure 4), but the combination of one or two poly I:CLC treatments with the single +6 hr Ab treatment was significantly more protective than the single drug controls (Figure 4).

A second protocol was designed to determine whether an early post SF infection therapy at a peripheral site (+6 hr, i.p.) in combination with a late intervention at the target organ (+96 hr, i.c.) could offer significant advantages. The rationale for this design modification was to insure that the therapeutic drugs were crossing the blood-brain barrier. The experimental design is explained in Table 17. In these experiments, combination therapy was significantly more protective than low dose (1000 U i.p. at +6 hr; 500 U i.c. at +96 hr) Ab therapy; while no significant difference was seen with high dose (2000 U i.p. at +6 hr; 500 U i.c. at +96 hr) Ab therapy (Figure 5).

F. Development of lasting immunity in SF-recovered mice

Mice which were successfully rescued from SF infection by combination therapy with poly I:CLC and anti-SF were examined for the development of lasting immunity. Mice were held for three weeks following the end of the experiment and rechallenged. Age-matched mice were used as controls. The challenge virus was either a second inoculum of the same virus (SF) used in the initial experiment, BZ, or LAC. It was expected that neither of the heterologous viruses would be affected by endogenous anti-SF antibodies which had been produced by the mice in response to the initial SF challenge. Results are given in Table 18. As expected, surviving of a previous challenge with SF gave complete immunity to a rechallenge with the same virus. Also as expected, no cross protection was seen upon rechallenge with the bunyavirus LAC, which is completely unrelated to SF. In fact, the "SF-survivor" mice fared significantly worse than did the controls in the LAC experiment. The significance, if any, of this observation is not clear at this time.

Sixty-one of 64 SF recovered mice that were subsequently challenged with BZV died. This result is consistent with reports of no crossreactivity between anti-SF Ab and flaviviruses. This is essentially equal to the historical survival rate for a 3LD75 BZV challenge in our laboratories. Thus survival of infection with SF imparts no protection to a subsequent challenge with BZV in recovered mice.

Discussion and Conclusions

In summary, this series of ongoing experiments has provided evidence which support the following conclusions.

1. Exogenous interferon appears to be ineffective in treating PIC infections, at least at levels up to 3×10^6 I.U./animal. Similarly, although the IFN inducer CL246,738 is active in guinea pigs, it is not an effective anti-PIC agent in these animals. These findings are consistent with previous reports of high resistance of arenaviruses to the antiviral action of interferon.

2. The immunomodulator AM-3 appears to be ineffective against PIC infections.

3. Rimantidine alone does not cure, but does extend the mean survival time of PIC-infected guinea pigs. It thus may be a promising agent for use in combination trials.

4. Ribavirin is an effective anti-PIC drug when a prolonged treatment regimen is used. Twice daily ribavirin therapy is superior to daily treatment, suggesting that continuous, controlled release regimens may prove optimal.

5. Guinea pigs which survive PIC infections through the use of antiviral agents develop a lasting immunity to reinfection with this agent.

6. IFN-α/β is effective against alpha-, flavi-, and bunyaviruses when used prophylactically. Effective IFN doses are lower in SF infections than in BZ, WN, LAC, or BW infections.

7. IFN-γ is a much less effective antiviral than IFN-α/β in all systems tested.

8. IFN-α/β inducers are potent antivirals when used prophylactically in alpha-, flavi-, and bunyavirus infections. LAC, BW, and SF viruses are particularly responsive to poly I:CLC and CL246,738.

9. The immunomodulators Ge-089, Ge-132, muramyl dipeptide, unencapsulated muramyl tripeptide, liposome encapsulated muramyl tripeptide, AM-3, and mannozyme are ineffective or inconsistent antivirals when used prophlyactically against SF and BZ infections.

10. The polyclonal B cell activator anti-mouse μ chain [$F(ab')_2$ fragment] is capable of activating splenic B cells *in vitro*. It does not consisently protect mice against SF or BZ infections at the doses used here, however.

11. Ribavirin and IFN-α/β interact synergistically against BZ, SF, and LAC infections.

12. Ribavirin and IFN-γ do not interact synergistically against BZ and SF infections when a single dose of up to 3000U IFN is used. Higher, more frequent doses of IFN-γ may be effective in combination with ribavirin, however.

13. Ribavirin and poly I:CLC do not interact positively against BZ, but give enhanced protection compared to single drug controls in the SF system.

14. Ribavirin and CL246,738 do seem to interact positively against BZ; this effect is not seen in the SF system.

15. The efficacy of combination antiviral treatments cannot be easily forecast from one virus system to another, as seen in conclusions 13 and 14.

16. IFN-α/β interacts synergistically with TNF-α, lowering the viremia from a BZ infection.

17. IFN-α/β has a positive, but inconsistent, interaction with IL-2 against BZ.

18. Poly I:CLC and antibody react synergistically to reduce mortality from SF and BZ infections when given therapeutically, at the stage of maximum viremia. Protection decreases markedly when virus has reached the brain from an i.p. infection.

19. Poly I:CLC and antibody react synergistically to reduce mortality from i.c. SF infections, even when given distally from the point of infection after a single virus multiplication cycle has been allowed to occur.

20. Mice surviving SF infection develop a lasting immunity to reinfection with SF. This immunity provides no protection to subsequent BZ of LAC challenges.

Table 1

Lack of effect on Pichindé infection by treatment with recombinant interferon-α A

Treatment[1]	No. animals	% Mortality	Mean Day of Death (±1 S.D.)
556 U	8	100	13.6 ± 1.1
1667 U	8	100	13.6 ± 1.1
16,667 U	4	100	13.7 ± 0.96
166,667 U	4	100	14.0 ± 0.81
Virus only	12	100	13.9 ± 0.79
Uninfected Interferon Treated 1667 u.	6	0	-

[1] Interferon treatments are shown as guinea pig equivalent units, as determined against vesicular stomatitis virus on primary guinea pig cells. Actual international units of IFN given in each experiment are 18-fold higher, i.e., 166,667 g.p. units = 3×10^6 I.U. rHuIFN-αA. IFN was administered as a single i.p. injection one hour prior to virus challenge.

Table 2

Lack of effect on Pichindé infection by treatment with CL246,738

Treatment	No. animals	% Mortality	Mean Day of Death (±1 S.D.)
15 mg/kg	8	100	14.3 ± (0.46)
5 mg/kg	8	100	14.6 ± (0.52)
Virus Only	8	100	15.7 ± (0.82)
Uninfected 15 mg/kg	6	0	-

Table 3

Effect of extended ribavirin treatment on Pichindé infection

Treatment	No. animals	% Mortality	Mean Day of Death (±1 S.D.)
25mg/kg/day 14 days	8	100	22.5±2.8[1]
25 mg/kg/day 28 days	12	25[2]	21.0 ± 2.0[3]
Virus Only	7	100	15.6 ± 4.6
Drug Only 25mg/kg/day 28 days	3	0	-

(1) Student's t-test $p = 0.005$ compared to untreated animals.

(2) $X^2 = 9.98$ $p = 0.002$ compared to untreated animals.

(3) Student's t-test $p = 0.02$ compared to untreated animals.

Table 4
Effect of MuIFN-α/β on Arbovirus Infections

Virus		Dose (U/mo)	% MRT	% Protection Relative to Virus Control
La Crosse	Experiment #1	300	100.0	0.0
		1,000	40.0	48.5
		3,000	50.0	35.6
		10,000	60.0	22.7
		30,000	60.0*	22.7
		100,000	0.0*	100.0
		None	77.7	NA
	Experiment #2	30,000	10.0*	90.0
		100,000	10.0*	90.0
		300,000	0.0*	100.0
		None	100.0	NA
Bunyamwera		30,000	20.0	50.0
		100,000	20.0*	50.0
		300,000	0*	100.0
		None	40.0	NA
West Nile		300	22.2	50.0
		;,000	44.0	0.0
		3,000	55.0	0.0
		10,000	25.0	43.6
		None	44.4	NA

Drug Schedule: - 2 hrs, i.p.
Nominal Virus Dose: 1 LD_{50}
No. Mice/Cell: 8-10
*Fisher's exact test p. $\leq$ 0.05 compared to virus control mortality.

Table 5

Titration of rHuIFN-α A/D Against SFV

Hu IFN-α A/D (U/mo)	Time of Administration (hrs)	% Mortality	% Protection relative to virus control
1×10^4	-2	55.4	30.7
3×10^3	-2	30.0*	62.5
1×10^3	-2	30.0*	62.5
3×10^2	-2	50.0	37.5
1×10^2	-2	80.0	0
1×10^3	-2, +24, +48 +72	100.0	0
Virus Control	-	80.0	NA

3 LD_{75} SFV/mouse; 10 mice per group.
* Fisher's exact test $p. \leq 0.05$ compared to virus control mortality.

Table 6

Effect of Poly I:CLC on Arbovirus Infections

Virus		Dose (μg/mo)	% MRT	% Protection Relative to Virus Control
Bunyamwera	Experiment #1	2.4	10	66.6
		0.8	10	66.6
		0.24	0	100.0
		0.08	0	100.0
		None	30	NA
	Experiment #2	8.0	0	100.0
		2.4	0	100.0
		0.8	0	100.0
		0.24	0	100.0
		None	25	NA
La Crosse	Experiment #1	2.4	0	100.0
		0.8	10	50.0
		0.24	0	100.0
		0.08	10	50.0
		None	20	NA
	Experiment #2	8.0	12.5	50.0
		2.4	0	100.0
		0.8	0	100.0
		0.24	0	100.0
		None	25	NA
West Nile		2.4	0.0*	100.0
		0.8	11.1*	80.0
		0.24	11.1*	80.0
		0.08	22.2	60.0
		0.024	22.2	60.0
		None	55.5	NA

Drug Schedule: - 6 hrs, i.p.
Nominal virus dose: 1 LD_{75}
No. mice/cell: 8-10
Medium for drugs: HBSS
* Fisher's exact test $p. \leq 0.05$ compared to virus control mortality.

Table 7

Effect of CL246,738 on Bunyavirus Infections in Weanling Mice

Virus	Drug Dose CL246,738 (mg/mo)	% MRT	% Protection Relative to Virus Control
Bunyamwera	0.1	20*	50
	0.3	0*	100
	1.0	0*	100
	3.0	0*	100
	None	40	NA
La Crosse	0.1	0*	100
	0.3	0*	100
	1.0	0*	100
	3.0	0*	100
	None	50	NA

Drug Schedule: - 24 hrs., per os
Nominal virus dose: 1 LD_{75}
No. mice/cell: 10
Medium for drugs: P.D.
* Fisher's exact test p. $\leq$ 0.05 compared to virus control mortality.

Table 8

Serum interferon induced by oral Ge-132 in weanling ICR mice

Oral Dose	Animal No.	Serum IFN at time after drug administration	
		24 hr	48 hr
300 mg/kg	1	<30	2,000
	2	700	700
	3	<30	300
	4	2,000	200
100 mg/kg	5	<30	300
	6	700	<30
	7	2,000	<30
	8	1,000	N.D.
30 mg/kg	9	<30	<30
	10	<30	<30
	11	<30	<30
	12	<30	<30
10 mg/kg	13	<30	N.D.
	14	300	200
	15	<30	300
	16	1,000	<30
3 mg/kg	17	<30	<30
	18	<30	<30
	19	<30	<30
	20	<30	<30

Table 9

Effect of low doses of Enf CDJ 38 against Bunyavirus infections of weanling mice

Virus	Dose (U/injection i.p.)	Schedule (Hr.)	% MRT	% Protection Relative to Virus Control
Bunyamwera	128	0	90	0
	128	-2,0	90	0
	128	-2,0,+2	90	0
	128	-2,0,+24	70	0
	None	0	60	NA
La Crosse	128	0	40	50
	128	-2,0	60	25
	128	-2,0,+2	80	0
	128	-2,0,+24	60	25
	None	0	80	NA

Nominal Virus Dose: 1 LD_{75}, i.p.
No. Mice/Cell: 10

Table 10

Effect of goat anti-mouse μ $F(ab')_2$ on Banzi virus infection

	Drug Dose per Injection (μg/mo)	% MRT	% Protection Relative to Virus Control
Expt. 1	40	37.5*	62.5
	None	100	NA
Expt. 2	80	87.5	12.5
	40	87.5	12.5
	None	100	NA
Expt. 3	80	100	0
	40	100	0
	None	100	NA

Drug Schedule: - 48, - 24 hrs., i.p.
Nominal Virus Dose: 3 LD_{75}
No. Mice/Cell: 8 (Expt. 1,2); 10 (Expt. 3)
Medium for Drug: EMEM
* Fisher's exact test $p. \leq 0.05$ compared to virus control mortality.

Table 11

Effect of combination therapy with Ribavirin and MuIFN-α/β against Semliki Forest and La Crosse viruses

Virus		Dose/injection Ribavirin (μg/mo)	Dose/injection MuIFN-α/β (U/mo)	% MRT	% Protection Relative to Virus Control
Semliki Forest		800	1000	87.5	12.5
		800	3000	75.0	25.0
		None	1000	100.0	0.0
		None	3000	62.5	37.5
		800	None	100.0	0.0
		None	None	100.0	NA
La Crosse	Expt. #1	800	30,000	0*	100
		800	60,000	0*	100
		None	30,000	25	24.9
		None	60,000	23	30.9
		800	None	25	24.9
		None	None	33.3	NA
	Expt. #2	800	30,000	11.1	66.6
		800	60,000	0.0	100.0
		None	30,000	33.3	0.0
		None	60,000	0.0	100.0
		800	None	22.2	33.3
		None	None	33.3	NA

Drug Schedule: Ribavirin: - 24, 0, +24, +48, +72 hrs; i.p.
IFN-α/β: -2 hr; i.p.
Nominal virus dose: 3 LD_{75} for SF; 1 LD_{75} for LAC
No. Mice/Cell: 8-12
*Fisher's exact test p. $\leq$ 0.05 compared to virus control mortality.

Table 12

Combination treatment of Semliki Forest virus with Poly I:CLC and Ribavirin

Poly I:CLC (mg/mo)	Ribavirin (μg/mo)	% Mortality	% Protection relative to virus control
Experiment #1			
0.27	800	11.1*	85.7
0.27	None	44.4*	42.8
0.08	800	11.1*	85.7
0.08	None	80	0
0.04	800	55.5	28.5
0.04	None	77.7	0
None	800	77.7	0
None	None	77.7	N/A
Experiment #2			
0.27	800	90	0.0
0.27	None	80	11.1
0.08	800	70	22.2
0.08	None	60	33.3
None	800	90	0.0
None	None	90	N/A

Drug Schedule: Ribavirin - 24, -1, +24, +48, +72 hrs.
Poly I:CLC - 6 hrs.
Nominal Virus Dose: 3 LD_{75}
No. Mice/Cell: 10 (Experiment #2); 9 (Experiment #1)
*Fisher's exact test $p. \leq 0.05$ compared to virus control mortality.

Table 13

Combination treatment of Arbovirus Infections with CL246,738 and Ribavirin

Virus	CL246,738 (mg/mo)	Ribavirin (µg/mo)	% Mortality	% Protection relative to virus control
Banzi	1.0	800	40*	60
	1.0	None	60*	40
	0.3	800	10*	90
	0.3	None	70	30
	0.1	800	70	30
	0.1	None	80	20
	None	800	100	0
	None	None	100	N/A
Semliki Forest	1.0	800	30*	70
	1.0	None	10*	90
	0.3	800	90	10
	0.3	None	80	20
	0.1	800	90	10
	0.1	None	70	30
	None	800	90	10
	None	None	100	N/A
La Crosse	0.3	800	11.1	55.5
	0.3	None	0	100
	0.1	800	0	100
	0.1	None	0	100
	None	800	0	100
	None	None	25	N/A

Drug Schedule: Ribavirin: -24, -1, +24, +48, +72 hrs.; i.p.
CL246,738: -24; per os.
Nominal Virus Dose: 1 LD_{75} (LAC); 3 LD_{75} (BZ,SF)
No. Mice/Cell: 8-10
Medium for drugs: HBSS for ribavirin; EMEM for CL246,738
*Fisher's exact test p. $\leq$ 0.05 compared to virus control mortality.

Table 14

Late combination therapy against Semliki Forest virus Infection with Poly I:CLC and anti-SF rabbit antibodies

Time of therapeutic Intervention	Drug Dose (i.p.) Poly I:CLC µg/Mo	Drug Dose (i.p.) anti-SF Ab U/Mo	% MRT	% Protection Relative to Virus Control
Experiment #1				
+48 hr	80	2000	50*	50
	80	1000	30*	70
	80	500	30*	70
	80	None	100	0
	None	2000	50*	50
	None	1000	50*	50
	None	500	100	0
	None	None	100	N/A
+72 hr	80	2000	100	0
	None	2000	100	0
	80	None	100	0
	None	None	100	0
Experiment #2				
+8 hr	80	1000	0*	100
	80	500	0*	100
	80	None	100	0
	None	1000	0*	100
	None	500	0*	100
	None	None	100	N/A
+24 hr	80	1000	41.6*	58.4
	80	500	75.0	25.0
	80	None	91.6	8.4
	None	1000	91.6	8.4
	None	500	100	0
	None	None	100	N/A
+48 hr	80	1000	50*	50
	80	500	100	0
	80	None	91.6	8.6
	None	1000	66.7*	33.4
	None	500	100	0
	None	None	100	N/A

Nominal Virus Dose: 3 LD_{75}
Number Mice/Cell: 10-12
*Fisher's exact test p. $\leq$ 0.05 compared to virus control mortality.

Table 15

Timed therapy with poly I:CLC and rabbit anti-SF antibodies against intracranial SF infection

Time Administered (Hrs)	Drug Dose: Poly I:CLC (µg/mo)	Drug Dose: Anti-SFV Ab (U/mo)	% MRT	% Protection Relative to Virus Control
-15	80	1000	37.5*	62.5
0	80	1000	87.5*	12.5
+6	80	1000	50.0*	50.0
+24	80	1000	87.5	12.5
Virus Control	None	None	100	N/A
Drug Control	80	1000	0	N/A

Drug Schedule: As shown above, time of administration is relative to virus challenge at 0 hr. Drugs administered i.p.
Nominal Virus Dose: 3 LD_{75}
Number Mice/Cell: 8
Medium for Drug: EMEM
*Fisher's exact test p. $\leq$ 0.05 compared to virus control mortality.

Table 16

Late therapy with Poly I:CLC and rabbit anti-SF antibodies for intracranial SF infection

	Drug Schedule (hr)			
	Poly I:CLC (80 µg/dose)	Anti-SFV Ab (U/mo)	% MRT	% Protection Relative to Virus Control
Experiment #1		1000 U at:		
	+6	+6	55.5*	44.5
	+6,96	+6	37.5*	62.5
	None	+6	100	0
	+6	+6,+96	50*	50.0
	+6,+96	+6,+96	44*	56.0
	None	+6,+96	100	0
	+6	None	100	0
	+6,+96	None	100	0
	None	None	100	N/A
Experiment #2		1000 U at:		
	+6	+6	50*	50
	+6,+96	+6	30*	70
	None	+6	100	0
	+6	+6,+96	55.5*	44.5
	+6,+96	+6,+96	40*	60
	None	+6,+96	100	0
		2000 U at:		
	+6	+6	50*	50
	+6,+96	+6	30*	70
	None	+6	100	0
	+6	+6,+96	10*	90
	+6,+96	+6,+96	20*	80
	None	+6,+96	80	20
	+6	None	100	0
	+6,+96	None	100	0
	None	None	100	N/A

Drug Route: i.p.
Drug Schedule: As shown, relative to virus challenge at 0 hr.
Nominal Virus Dose: 3 LD_{75}
Number Mice/Cell: 8-10
Medium for Drug: EMEM
* Fisher's exact test p. $\leq$ 0.05 compared to virus control mortality.

Table 17

Late i.p. and i.c therapy with Poly I:CLC and anti-SFV rabbit antibodies for intracranial SF infection

Drug							
Poly I:CLC (μg/mo)	Route	Time	Ab (U/mo)	Route	Time	% MRT	% Protection Relative to Virus Control
80	i.p.	+6	1000	i.p.	+6	20*	80
20	i.c.	+96	500	i.c.	+96		
	None		1000	i.p.	+6	75	25
			500	i.c.	+96		
80	i.p.	+6	2000	i.p.	+6	70	30
20	i.c.	+96	500	i.c.	+96		
	None		2000	i.p.	+6	70	30
			500	i.c.	+96		
80	i.p.	+6		None		100	0
20	i.c.	+96					
	None			None		100	N/A

Drug Schedule: As shown in drug column
Nominal Virus Dose: 3 LD_{75}
Number Mice/Cell: 10
Medium for Drug: EMEM
* Fisher's exact test p. $\leq$ 0.05 compared to virus control mortality.

Table 18

Immune status of mice surviving SF infection after receiving late therapy with poly I:CLC and rabbit anti-SF antibodies

Immune Status	No.	Rechallenge Virus	% MRT	% Protection Relative to Virus Control
Recovered	24	Semliki Forest	0*	100
	64	Banzi	95.3	4.7
	9	La Crosse	78	0
Unexposed Controls	22	Semliki Forest	100	N/A
	24	Banzi	100	N/A
	10	La Crosse	10	N/A

Nominal rechallenge virus dose: 3 LD_{75} (SF; BZ); 1 LD_{75} (LAC)
*Fisher's exact test $p. \leq 0.05$ compared to virus control mortality.

Figure Legends

Figure 1. Treatment of Pichinde' virus infection with rimantidine. Strain 13 guinea pigs [500 g average body weight] were injected with 3.0 mg/kg rimantidine in Hank's Balanced Salt Solution, intraperitonally, on days 0,1,2,3,4,7,9, and 11 relative to virus challenge with 10^4 PFU Pichinde' on day 0. Cumulative percent mortality is graphed. Rimantidine treatment extended mean survival from 12.5 to 15.2 days after infection.

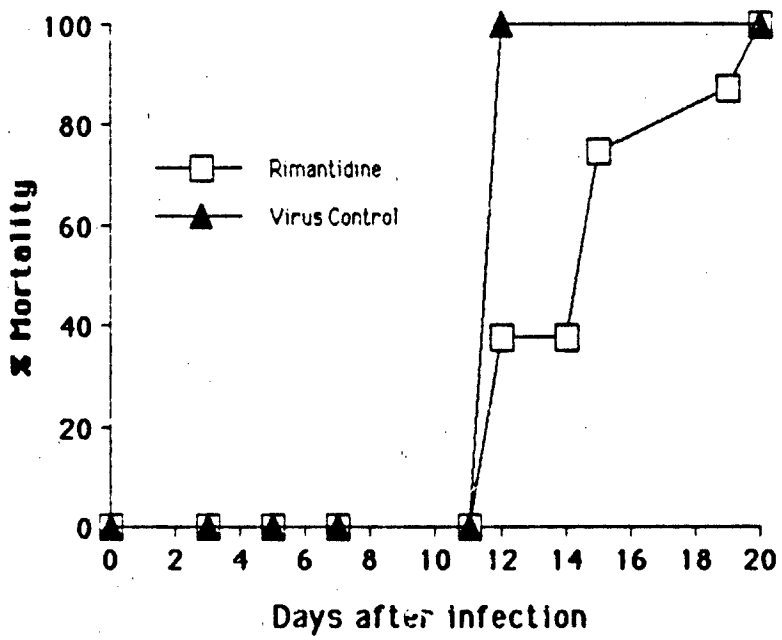
Figure 2. Reduction of Banzi virus viremia by combination treatment using IFN-α/β and rTNF-α. MoIFN-α/β (10,000 U/mo) was injected i.p. 2 hr before virus challenge. rTNF-α (3μg/mo/injection) was injected i.p. at -24,0,24,48, and 72 hr relative to virus challenge with 3LD75 Bz. Mice (5/group) were bled from the retro-orbital sinus on days 2,3,4, and 6. BZ in serum was titered on CER cells. BZ viremia was significantly reduced by the combination treatment compared to single drug and virus controls.

Figure 3. Combination treatment of intracranial Semliki Forest virus infection with anti-SF antibody and poly I:CLC. Weanling mice were infected with 3LD75 SF i.c. at time 0. Treatment with 1000 U anti-SF Ab and/or 80 μg poly I:CLC was administered i.p. at 6 or at 6 and 96 hr post-infection. All combination treatments significantly reduced mortality compared to placebo and single drug controls.

Figure 4. Combination treatment of intracranial Banzi virus infection with anti-BZ antibody and poly I:CLC. Weanling mice were infected with 3LD75 BZ i.c. at time 0. Treatment with 1000 U anti-BZ Ab and/or 80 μg poly I:CLC was administered i.p. at 6 or at 6 and 96 hr post-infection. Top Panel. Anti-BZ Ab administered at +6 and +96 hr. Addition of poly I:CLC does not significantly alter mortality compared to antibody controls. Bottom Panel: Anti-BZ Ab administered at +6 hr only. Combination treatment significantly lowers mortality compared to single drug and placebo controls.

Figure 5. Combination intraperitoneal and intracranial therapeutic treatment of intracranial Semliki Forest virus infection with anti-SF antibody and poly I:CLC. Weanling mice were infected with 3LD75 SF i.c. at time 0. Tretment with 1000 U anti-SF Ab and/or 80 μg poly I:CLC was administered i.p. at 6 hr post-infection. Treatment with 500 U anti-SF Ab and/or 20 μg poly I:CLC was administered i.c. at 96 hr post-infection. Placebo controls received saline only. Combination treatment significantly lowers mortality compared to single drug and placebo controls.

Figure 1. Treatment of Pichinde' virus infection with rimantidine.



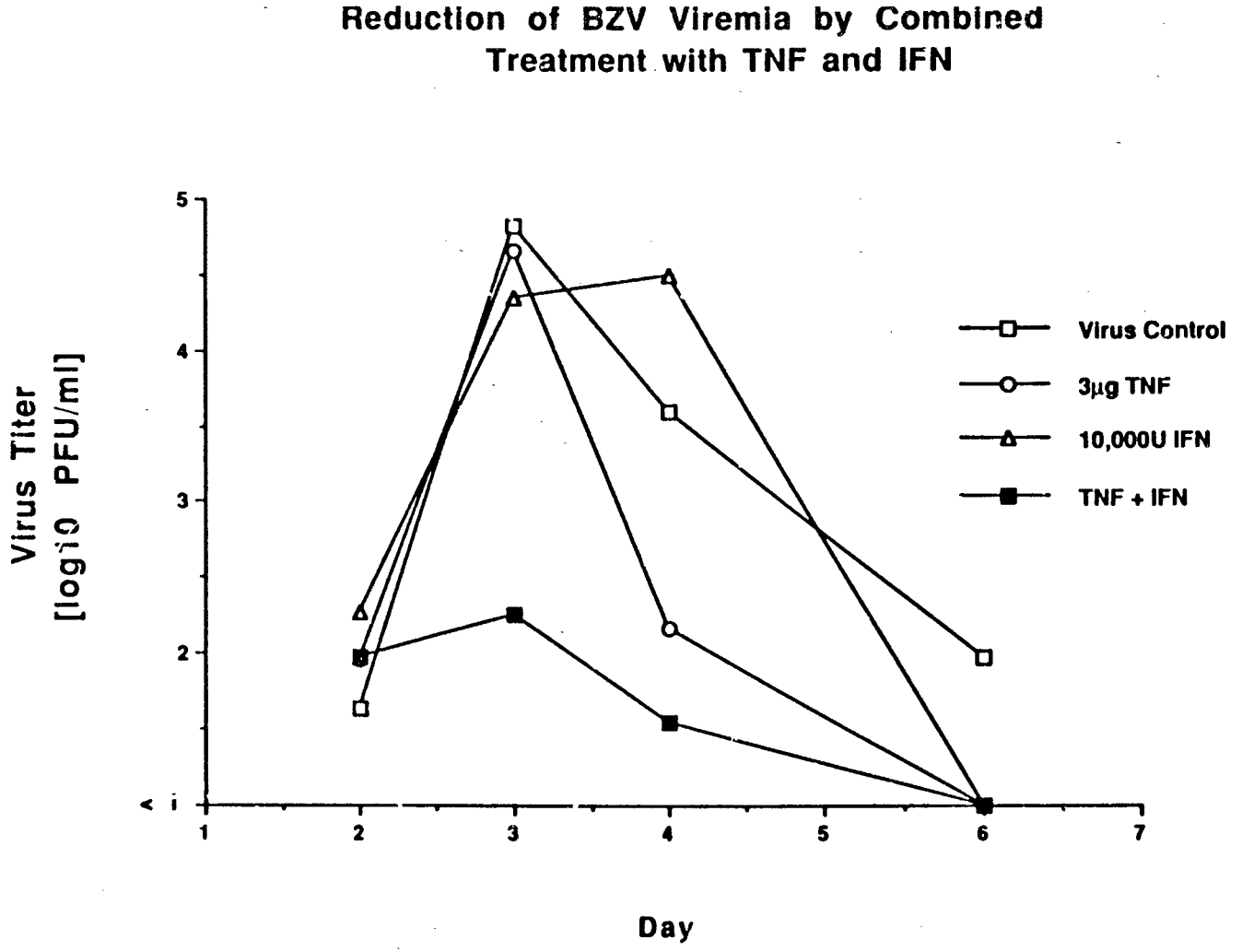


Figure 2. Reduction of Banzi virus viremia by combination treatment using IFN-α/β and rTNF-α.

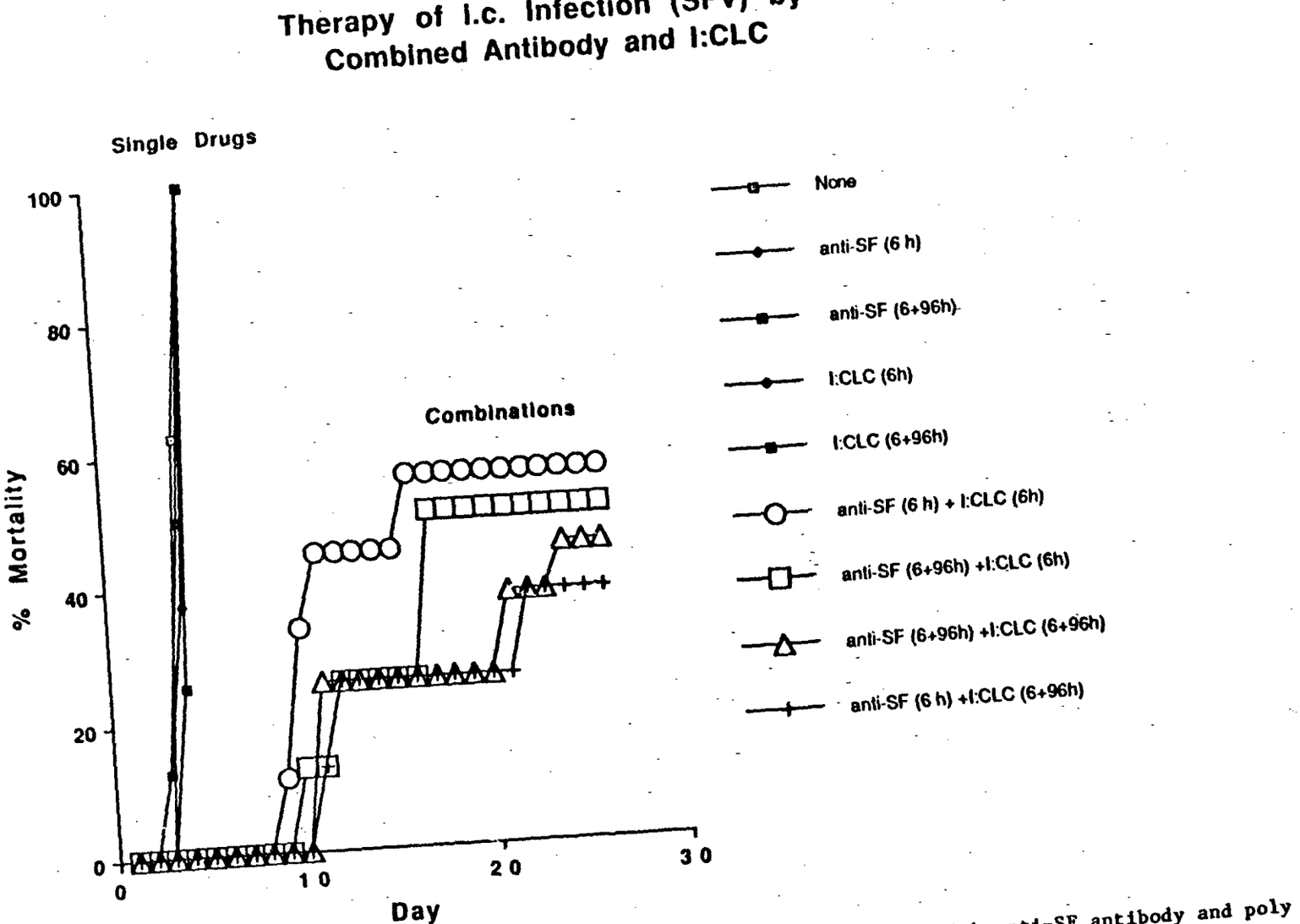


Figure 3. Combination treatment of intracranial Semliki Forest virus infection with anti-SF antibody and poly I:CLC.

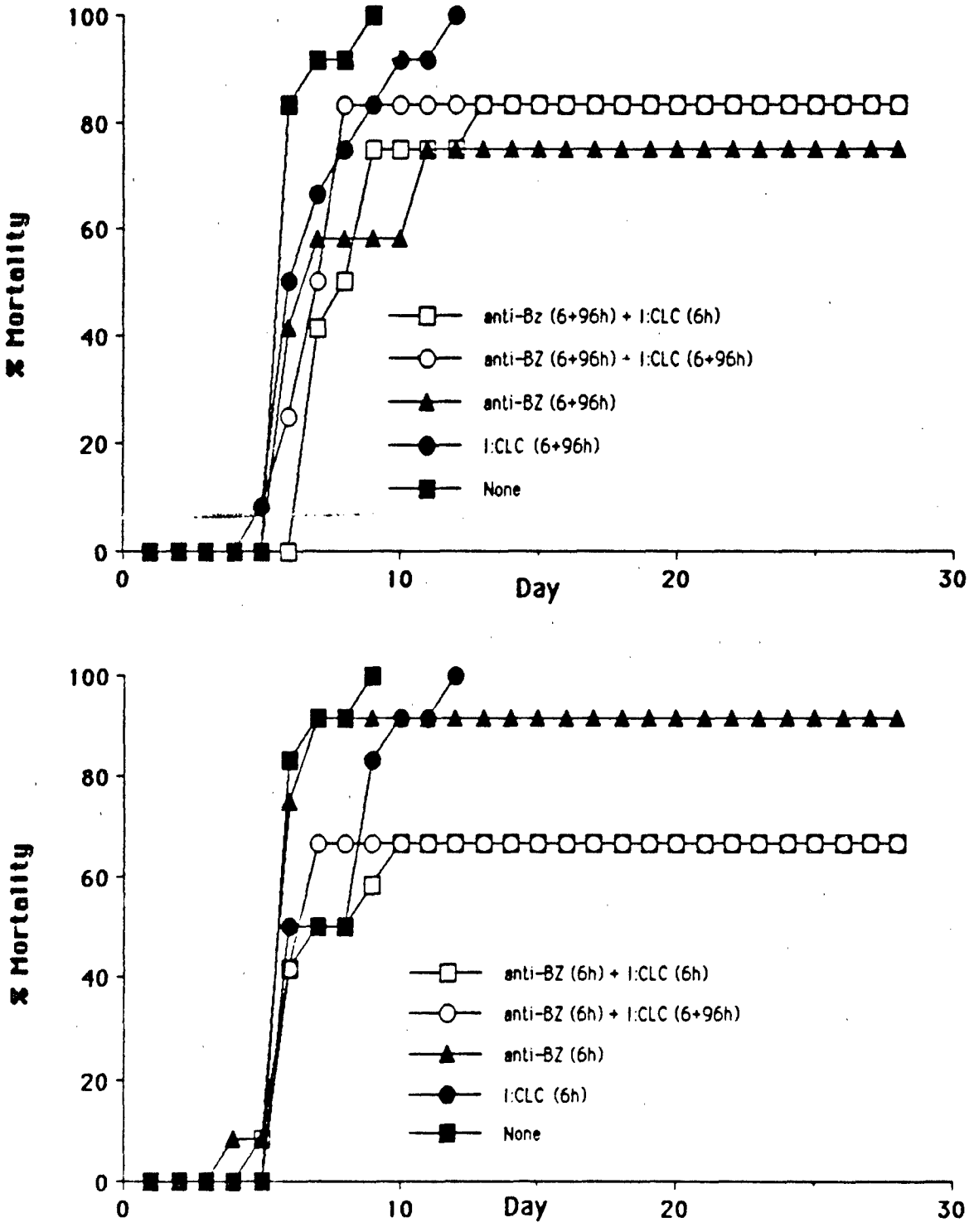
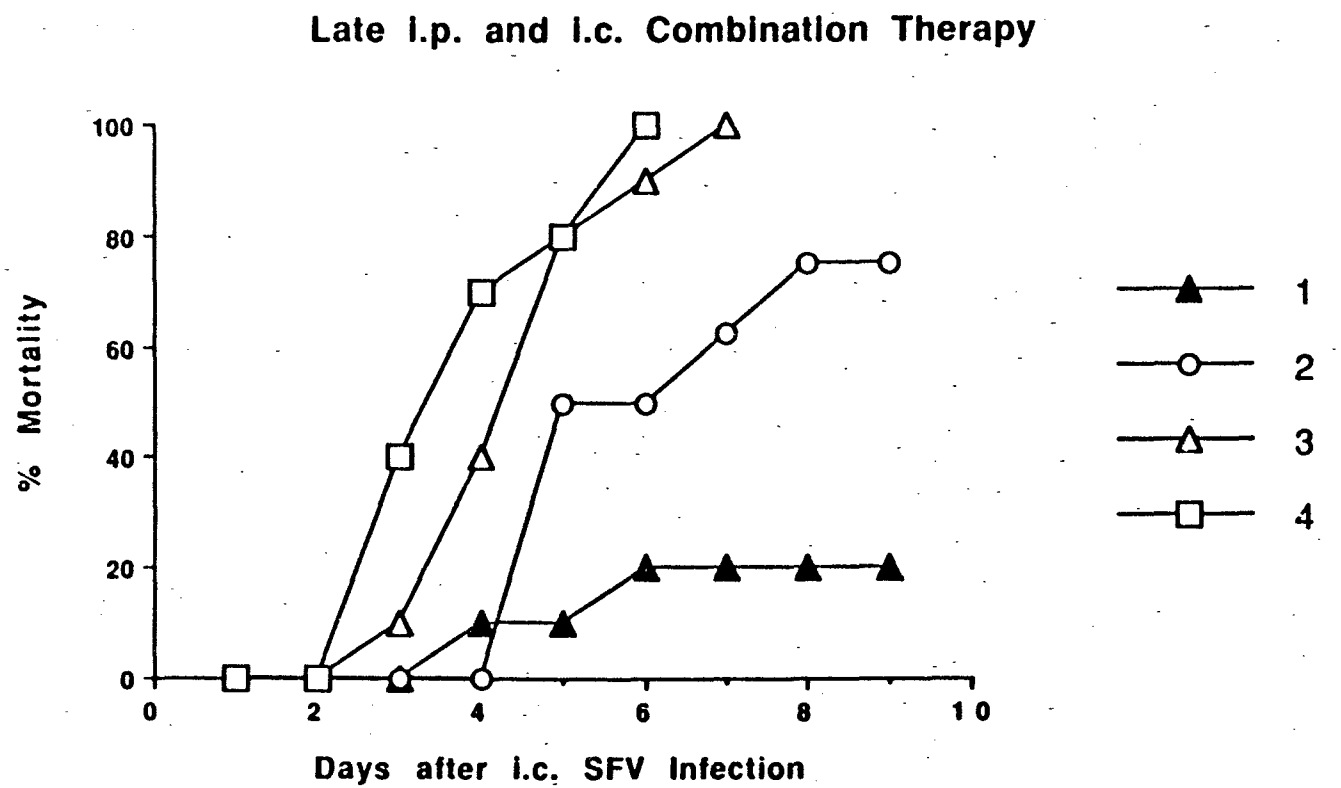


Figure 4. Combination treatment of intracranial Banzi virus infection with anti-BZ antibody and poly I:CLC.



1) 1000 U anti-SF + 80 ug I:CLC i.p. (6h) then 500 U anti-SF + 20 ug I:CLC i.c. (96h)

2) 1000 U anti-SF i.p. (6h) then 500 U anti-SF i.c. (96h)

3) 80 ug I:CLC i.p. (6h) then 20 ug I:CLC i.c. (96h)

4) Control

Figure 5. Combination intraperitoneal and intracranial therapeutic treatment of intracranial Semliki Forest virus infection with anti-SF antibody and poly I:CLC.